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PRINCIPAL INVESTIGATOR: Timothy P. Quinn, M.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Timothy P. Quinn, M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of California, San Francisco
San Francisco, California 94143-0962

E-Mail: tpquinn@itsa.ucsf.edu

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The purpose of this project is to use a novel chimeric cell death receptor (termed VEGFR2Fas) that is triggered by vascular endothelial growth factor (VEGF), as a means to kill prostate cancer cells and vascular endothelial cells.

The scope of this project involves: (i) creating adenoviral reagents to express the VEGFR2Fas receptor in prostate cancer cells and endothelial cells; (ii) determining if the VEGFR2Fas receptor kills cells in a VEGF-dependent manner; and (iii) identifying methods for increasing the killing activity of VEGFR2Fas.

The major findings to date include: (i) we have generated adenoviral reagents to express VEGFR2Fas as well as control adenoviruses; (ii) we have demonstrated that the adenoviral reagents direct expression of VEGFR2Fas or control receptors in the prostate cancer cell line DU145, and confirmed expression of the receptors by immunoblot; and (iii) we have demonstrated that expression of VEGFR2Fas in DU145 cancer cells activates apoptotic signaling and induces apoptosis in a very high percent of cancer cells within 72 hours.

This may be the first demonstration that cancer cells can be induced to undergo apoptosis by forcing an endogenous growth factor to function instead as a death factor.

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## Table of Contents

## INTRODUCTION

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The major findings to date include: (i) we have generated adenoviral reagents to express VEGFR2Fas as well as two control adenoviruses; (ii) we have demonstrated that the adenoviral reagents direct expression of VEGFR2Fas or control receptors in DU145 prostate cancer cells and endothelial cells, and have confirmed expression of the receptors by immunoblot; and (iii) we have demonstrated that expression of VEGFR2Fas in DU145 cancer cells activates apoptotic signaling and induces apoptosis in a very high percent of cancer cells within 72 hours.

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## BODY

### Statement of Work

**Task 1. Generate replication-defective adenovirus expressing VEGFR2Fas and appropriate control adenoviruses.**

**Task 1A: Subclone cDNAs for VEGFR2Fas and control receptors into adenoviral vector.**

We used the Adeno-X system from Clontech to generate adenoviruses (AdV). The procedure involves four steps: (i) subclone cDNA for protein of interest into a shuttle vector that contains the CMV promoter; (ii) remove cDNA with CMV promoter and adjacent sequences from shuttle vector, and ligate into an adenoviral vector; (iii) transfect adenoviral vector into 293 HEK cells; and (iv) recover adenovirus and confirm expression of protein of interest in target cells.

To make adenovirus that expresses VEGFR2Fas, the cDNA for VEGFR2Fas (which we had previously constructed) was subcloned into the shuttle vector, and then transferred to the adenoviral vector using standard molecular biology techniques. To make the two control adenoviruses, AdV / LacZ and AdV / CG-VEGFR2Fas, the cDNAs for LacZ and CG-VEGFR2Fas were similarly subcloned into the shuttle and adenoviral vectors. LacZ is the beta-galactosidase gene and is a widely used control protein. CG-VEGFR2Fas is identical to VEGFR2Fas except that it has a C-to-G point mutation in the Fas "death domain" that abolishes Fas apoptotic activity. CG-VEGFR2Fas is the optimal control protein to compare with VEGFR2Fas; when cells are killed by expression of VEGFR2Fas but not by expression of CG-VEGFR2Fas, non-specific killing mechanisms can be ruled out. Both VEGFR2Fas and CG-VEGFR2Fas have a hemagglutinin (HA) epitope tag on the carboxyl tail (see Figure 1) which we have used to detect expression of the receptors by Western blot.

Adenoviral plasmids with VEGFR2Fas, CG-VEGFR2Fas or LacZ sequences were grown up in DH5α bacteria and the plasmids were purified using Qiagen alkaline lysis maxiprep reagents. Task 1A was fully accomplished.

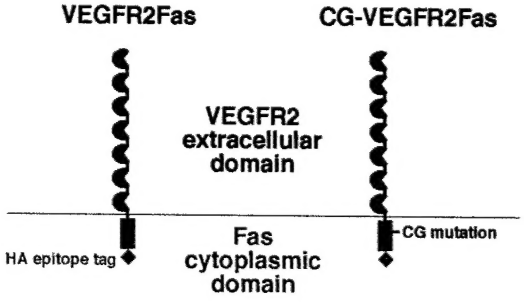


**Figure 1. VEGFR2Fas and CG-VEGFR2Fas receptors.**
Extracellular VEGF-binding domains of VEGFR-2 are shown in black, the cytoplasmic domain of Fas in red, and the carboxyl tail hemagglutinin tag (HA) in blue. CG-VEGFR2Fas has a C-to-G point mutation in the Fas cytoplasmic "death domain" that abolishes Fas apoptotic signaling.

**Task 1B: Transfect adenoviral cDNA into 293A cells and recover adenovirus.**

Adenoviral plasmids with VEGFR2Fas, CG-VEGFR2Fas or LacZ sequences were transfected into 293 cells by calcium phosphate-mediated transfection. Initially we could not generate significant amounts of AdV / VEGFR2Fas, and we suspected this might occur because the 293 cells were expressing and being killed by VEGFR2Fas before they were able to make the adenovirus. Therefore we revised the protocol to include the caspase inhibitor ZVAD (40 μM) in the 293 medium, which resolved the problem. After 7 – 10 days of culture, typical viral plaques were observed in the 293 cells, indicating adenoviral production. Adenovirus was recovered by freeze/thaw lysis of the 293 cells, and amplified by reinfection and recovery from 293 cells. The viral titer of AdV / VEGFR2Fas was determined to be 6.6 X $10^9$ viral particles/mL using an immunohistochemical assay kit from Clontech. The titer of AdV / CG-VEGFR2Fas was 2.2 X $10^9$ viral particles/mL, and the titer of AdV / LacZ was 6.3 X $10^9$ viral particles/mL. Task 1B was fully accomplished.

**Task 2. Use adenoviruses to express VEGFR2Fas or control viruses in prostate cancer cell lines and primary microvascular endothelial cells.**

**Task 2A: Transfect human prostate cancer cell lines and human microvascular endothelial cells in vitro with AdV/VEGFR2Fas or control adenoviruses. (Months 9-18)**
**Task 2B: Confirm that VEGFR2Fas protein is expressed in transfected cells by immunoblot techniques. (Months 9-18)**

To confirm that the AdV / VEGFR2Fas and AdV / CG-VEGFR2Fas viruses did in fact direct expression of the receptors, we initially infected porcine aortic endothelial (PAE) cells and examined cell lysates by Western blot for evidence of receptor expression. As seen in Figure 2, both adenoviruses successfully directed expression of their respective receptors, which were detected at their predicted size of ~140 kD.

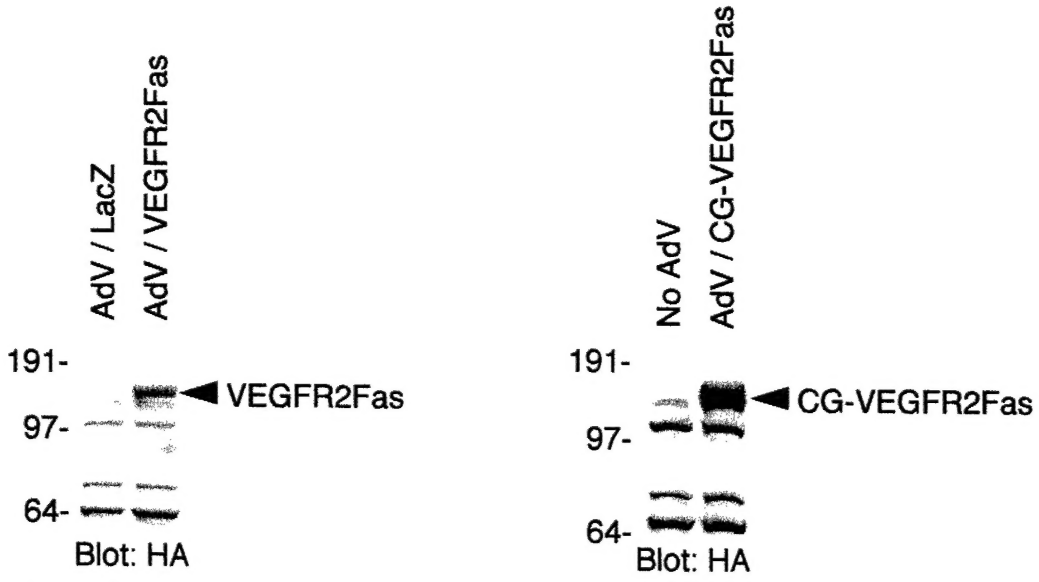


**Figure 2. Expression of VEGFR2Fas and CG-VEGFR2Fas receptors by adenoviral gene transfer.** To confirm that the adenoviruses work, adenoviruses containing VEGFR2Fas or CG-VEGFR2Fas cDNA sequences were put onto porcine aortic endothelial cells. Two days after infection, cells were lysed, electrophoresed by SDS/PAGE, and immunoblotted with antibody against the carboxyl tail HA epitope. Expression of both receptors is confirmed by the presence of the 140 kD band, which is the predicted size of the chimeric receptor protein.

We then confirmed that AdV / VEGFR2Fas and AdV / CG-VEGFR2Fas directed expression of their respective receptors in DU145 prostate cancer cells. DU145 cells growing in 6-well plates had 6 X $10^7$ viral particles of AdV / LacZ, AdV / VEGFR2Fas, or AdV / CG-VEGFR2Fas added to the medium. Two days later cells were lysed and Western blotted with HA antibody to detect expression of the HA-tagged receptors, or blotted with antibody against the Fas domain. As seen in Figure 3, both AdV / CG-VEGFR2Fas and AdV / VEGFR2Fas successfully directed expression of their respective receptors in DU145 cells at equivalent expression levels.

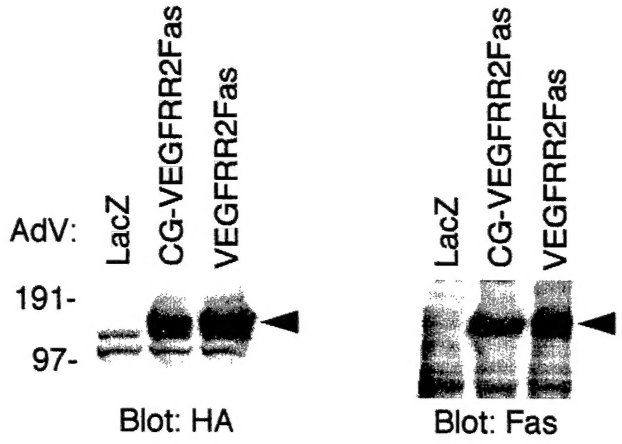


**Figure 3. AdV-directed expression of VEGFR2Fas and CG-VEGFR2Fas in DU145 prostate cancer cells.** AdV / VEGFR2Fas, AdV / CG-VEGFR2Fas, or the control virus AdV / LacZ were put onto DU145 prostate cancer cells. Two days after infection, cell lysates were immunoblotted with antibodies against the carboxyl tail HA epitope (left) or the Fas cytoplasmic domain. Similar expression levels of VEGFR2Fas and CG-VEGFR2Fas are demonstrated by the 140 kD bands.

We are currently extending these results to confirm that our adenoviruses express VEGFR2Fas and CG-VEGFR2Fas in PC3 and LNCaP prostate cancer cells, as well as human microvascular endothelial cells.

**Task 2C: For each prostate cancer cell line and endothelial cell type, characterize expression of VEGF, expression of apoptosis signaling or inhibiting proteins downstream of Fas, and sensitivity to endogenous Fas-mediated apoptosis. (Months 9-18)**

We have begun to characterize expression of VEGF in DU145 prostate cancer cells by Western blot analysis of cell lysates. Using an adenovirus that expresses human VEGF as a positive control, we have identified two commercially available antibodies that recognize human VEGF (both from Santa Cruz Biotechnology). We will use those antibodies to compare expression of VEGF in normal human prostate cells (from Clonetics) versus DU145, PC3, and LNCaP prostate cancer cells. We have also obtained antibodies against apoptosis effector proteins such as the uncleaved, zymogenic form of caspase-3 and caspase-9, and we will compare expression of those proteins in normal prostate cells versus the three prostate cancer cell lines.

**Task 3. Determine response of prostate cancer cell lines to expression of VEGFR2Fas.**

**Task 3A: Beginning with prostate cancer cell lines that express high levels of VEGF and are sensitive to Fas-mediated apoptosis, determine if expression of VEGFR2Fas stimulates apoptosis, using standard apoptosis assays.**

We had proposed that Task 3 would begin at 18 months, but because we were successful at generating adenoviral reagents more quickly than we anticipated, we have gone forward to investigate what happens when VEGFR2Fas is expressed in DU145 cells by AdV / VEGFR2Fas. As we described in the grant proposal, our hypothesis is that expression of VEGFR2Fas will stimulate apoptosis in prostate cancer cells that overexpress VEGF.

Using the adenoviruses, we expressed VEGFR2Fas or the controls CG-VEGFR2Fas or LacZ in DU145 prostate cancer cells. At 24, 48, and 72 hours after infection cells were lysed and Western blots performed to confirm receptor expression and to look for evidence of caspase activation using antibodies that specifically recognize the cleaved, active forms of several caspases. As seen in Figure 4, VEGFR2Fas expression in DU145 cells was initially detectable at 24 hours, and increased at 48 and 72 hours. CG-VEGFR2Fas expression followed a similar pattern with even higher receptor expression levels. Activation of caspase-3 and caspase-9 was found in cells expressing VEGFR2Fas, beginning at 24 hours after AdV / VEGFR2Fas infection, but no caspase activation was found in cells expressing the negative controls CG-VEGFR2Fas or LacZ.

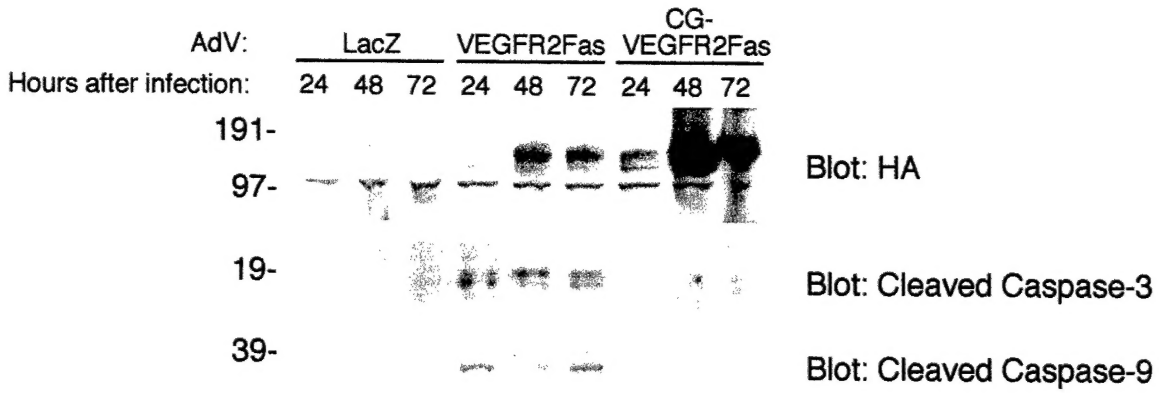


**Figure 4. Adenoviral expression of VEGFR2Fas in DU145 prostate cancer cells stimulates activation of apoptotic signaling.**
AdV / VEGFR2Fas, AdV / CG-VEGFR2Fas, or the control virus AdV / LacZ were put onto DU145 prostate cancer cells. Cells were lysed at 24, 48, or 72 hours, and electrophoresed by SDS/PAGE. Lysates were immunoblotted with HA antibody to demonstrate receptor expression, and with antibodies to the activated, cleaved forms of caspase-3 and caspase-9. Both caspase-3 and caspase-9 were activated in cells expressing VEGFR2Fas, but were not activated in the LacZ control cells or the cells expressing the CG-VEGFR2Fas receptor, which has an inactivating point mutation in the Fas death domain.

In a second, independent experiment (see Figure 5), we further demonstrated that expression of VEGFR2Fas in DU145 cells stimulates activation of caspase-8 (an initiator caspase) as well as cleavage of the caspase substrate PARP. The decreased expression of VEGFR2Fas on day 3, at a time when caspases are activated, likely represents cleavage of the VEGFR2Fas receptor itself.

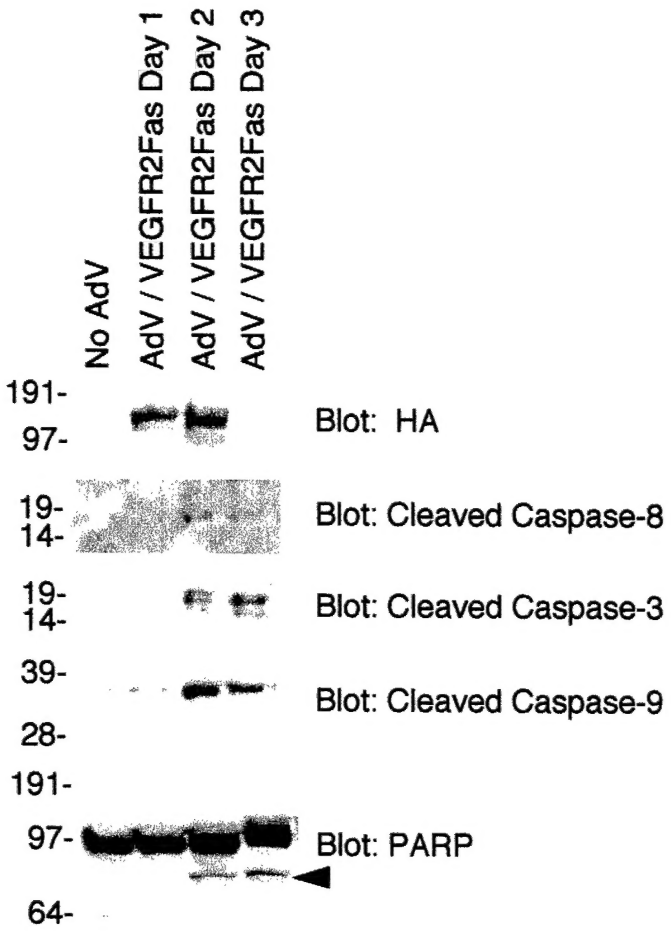


**Figure 5. Adenoviral expression of VEGFR2Fas in DU145 cells stimulates activation of caspases-8, -3, and -9, and cleavage of PARP.**
DU145 prostate cancer cells were infected with AdV / VEGFR2Fas or left uninfected as control (left lane). On days 1, 2, and 3 after infection cells were lysed and electrophoresed by SDS/PAGE. Immunoblot with HA antibody demonstrates expression of VEGFR2Fas beginning on day 1 after infection. Immunoblot with antibodies against the active, cleaved forms of caspase-8, caspase-3, and caspase-9 demonstrate activation beginning on day 2 after infection. Immunoblot with antibody against the caspase substrate PARP demonstrates cleavage of PARP (black arrowhead) beginning on day 2 after infection.

To determine the optimal amount of AdV / VEGFR2Fas to stimulate apoptotic signaling in DU145 cells, serial 10-fold dilutions of AdV / VEGFR2Fas were used to express VEGFR2Fas. Cells were lysed at 24, 48, and 72 hours after infection and Western blotted with antibodies to identify apoptotic signaling. As seen in Figure 6, caspase-3 and caspase-9 activation as well as PARP cleavage peaked at 48 hours after infection, and were highest in cells that received the

most AdV / VEGFR2Fas. These results demonstrate that VEGFR2Fas activates apoptotic signaling in a time-dependent and titer-dependent manner.

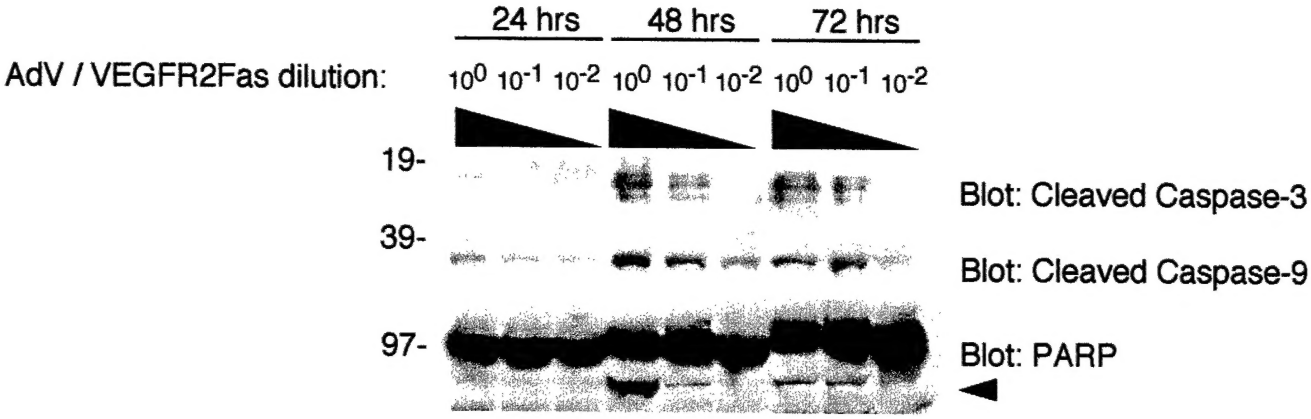


**Figure 6. Adenoviral expression of VEGFR2Fas in DU145 cells stimulates caspase and PARP cleavage in a time-dependent and titer-dependent manner.**
DU145 prostate cancer cells were infected with AdV / VEGFR2Fas at three titers (6.6 X 10^7, 6.6 X 10^6, and 6.6 X 10^5 virions/well) and lysed at 24, 48, or 72 hours after infection. Lysates were electrophoresed by SDS/PAGE. Immunoblot with antibodies against active, cleaved caspase-3 and caspase-9, and against the PARP substrate demonstrate that higher titers of AdV / VEGFR2Fas stimulate more activation of caspase-3 and caspase-9, and more cleavage of PARP. Peak activation occurs 48 hours after AdV / VEGFR2Fas infection.

Does activation of apoptotic signaling by VEGFR2Fas result in the death of DU145 cells? To examine this we used AdV to express VEGFR2Fas or the negative control receptor CG-VEGFR2Fas in DU145 cells, and examined them at 96 hours after infection. As seen in Figure 7, expression of VEGFR2Fas at the highest titer killed virtually all DU145 cells. Importantly, expression of CG-VEGFR2Fas did not kill the cells, demonstrating that cell death is due to Fas-mediated activation of apoptosis, and not due to adenoviral infection per se.

We would like to prove that expression of VEGFR2Fas is lethal only to cells that overexpress VEGF, such as cancer cells, and does not cause cell death in normal cells with low VEGF expression. To begin to address this question, we compared the responses of DU145 and PAE cells. PAE cells are a widely used endothelial cell line that were not derived from a tumor and which display growth characteristics in vitro similar to primary endothelial cell lines, e.g. contact-arrested growth. DU145 and PAE cells were infected with equivalent amounts of AdV / VEGFR2Fas or the control virus AdV / LacZ, or left untransfected as an additional negative control. As seen in Figure 8, by 54 hours after infection with AdV / VEGFR2Fas, DU145 cells were undergoing apoptosis, with evident membrane blebbing and cell loss. AdV / LacZ at the same titer did not stimulate apoptosis. In contrast to the DU145 cells, PAE cells infected with AdV / VEGFR2Fas showed no signs of apoptosis. This result is consistent with our published observation that PAE cells can express high levels of VEGFR2Fas and are not killed unless exogenous VEGF is added (reference 1). This result will be extended by examining the response of normal prostate cells and primary human endothelial cells to expression of VEGFR2Fas. It will be very exciting if expression of VEGFR2Fas is lethal only to prostate cancer cells.

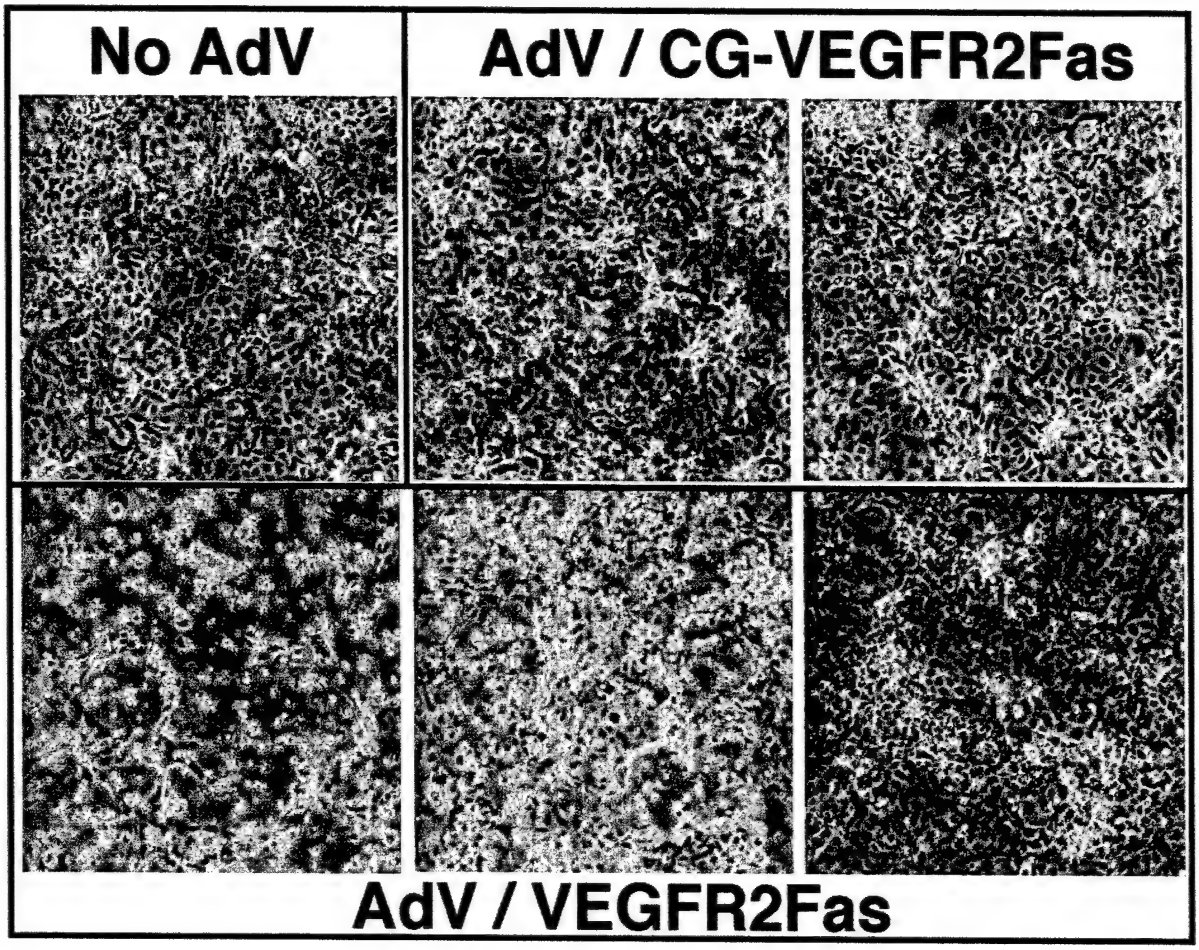


**Figure 7. Adenoviral expression of VEGFR2Fas in DU145 cells stimulates apoptosis.**
DU145 prostate cancer cells were infected with AdV / VEGFR2Fas at three titers (6.6 X 10^7, 6.6 X 10^6, and 6.6 X 10^5 virions/well, bottom panel left to right). Other cells were left uninfected (top left) or infected with AdV / CG-VEGFR2Fas (6.6 X 10^7 or 6.6 X 10^6 virions/well, top panel, middle and right). Cells left uninfected or expressing the inactive CG-VEGFR2Fas receptor remain normal. In contrast, cells expressing VEGFR2Fas undergo apoptosis with widespread cell death and fragmentation apparent in the cells that received the highest titer (bottom left). Less apoptosis is seen in the bottom middle panel, and essentially none in the bottom left panel, demonstrating that apoptosis is dependent upon the titer and expression of VEGFR2Fas.

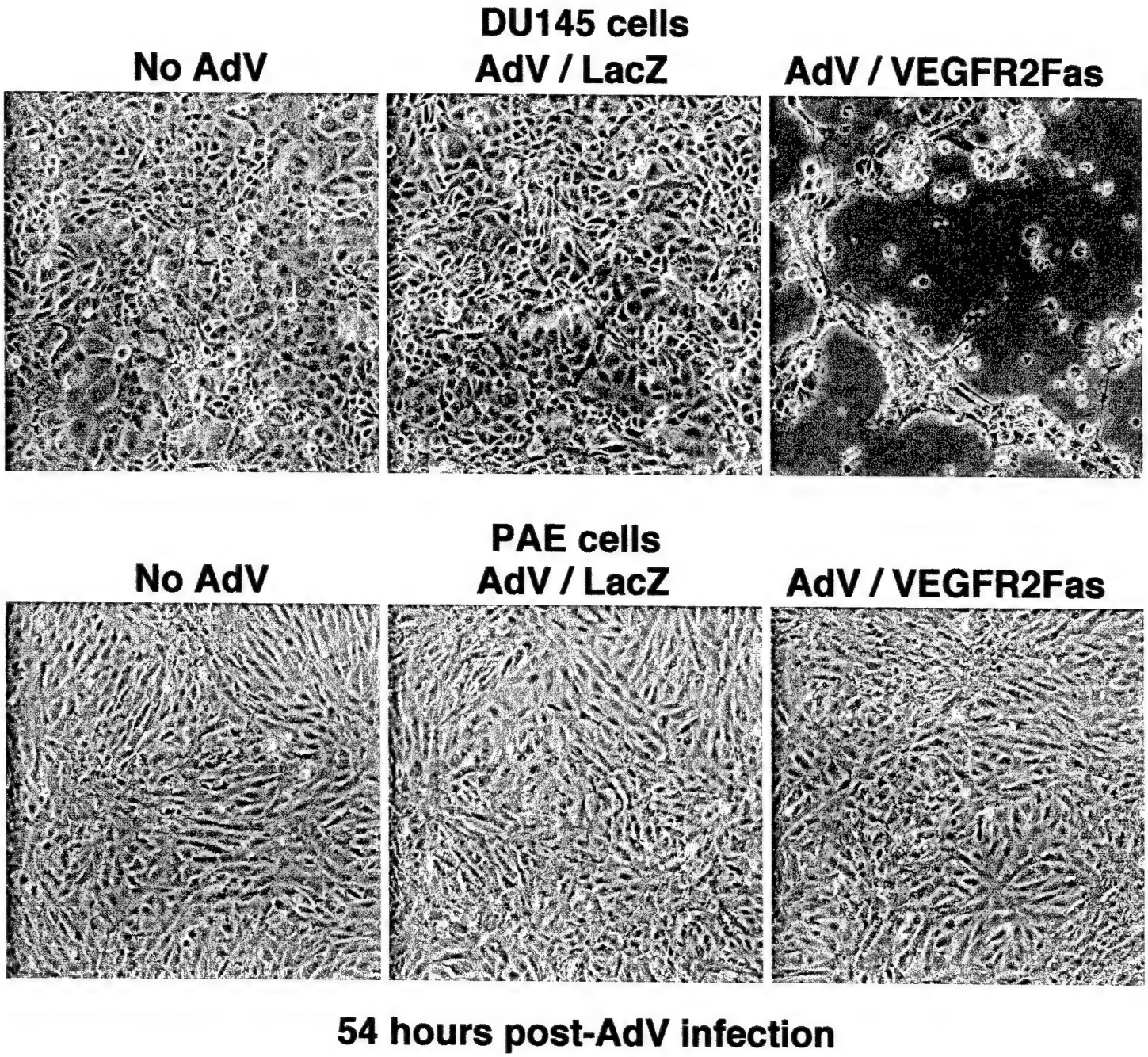


**Figure 8. Adenoviral expression of VEGFR2Fas kills DU145 prostate cancer cells but not porcine aortic endothelial cells.**
DU145 prostate cancer cells and porcine aortic endothelial cells (PAE) were infected with AdV / VEGFR2Fas or control AdV / LacZ at the same titers. As an additional negative control, other cells were left uninfected (No AdV, left panels). Cells left uninfected or expressing the control AdV / LacZ remained normal 54 hours after infection. DU145 cells expressing VEGFR2Fas demonstrate extensive apoptosis. In contrast, PAE cells expressing VEGFR2Fas remain normal, demonstrating that expression of VEGFR2Fas is lethal only to DU145 cells.

## KEY RESEARCH ACCOMPLISHMENTS

1. Created adenoviruses that express the VEGFR2Fas death receptor, and control adenoviruses that express the inactive control receptor CG-VEGFR2Fas or LacZ.
2. Demonstrated adenoviral-directed expression of VEGFR2Fas in DU145 prostate cancer cells and in endothelial cells.
3. Demonstrated that expression of VEGFR2Fas in DU145 cancer cells activates apoptotic signaling in a time- and titer-dependent manner.
4. Demonstrated that expression of VEGFR2Fas, but not the control receptor CG-VEGFR2Fas, stimulate apoptosis in DU145 prostate cancer cells.

## REPORTABLE OUTCOMES

1. The adenoviruses expressing VEGFR2Fas and CG-VEGFR2Fas will be shared with other interested investigators.
2. I anticipate that a manuscript and a new grant proposal based on this work will be submitted in the next few months.

## CONCLUSIONS

In summary, we have generated adenoviral reagents that direct expression of the VEGF-triggered death receptor VEGFR2Fas, and the inactive control receptor CG-VEGFR2Fas. We have used these adenoviral reagents to express VEGFR2Fas and the control receptor in DU145 prostate cancer cells, and have demonstrated that expression of VEGFR2Fas activates caspase signaling. Most importantly, we have found that expression of VEGFR2Fas in DU145 cells stimulates extensive killing. This may be the first demonstration that a growth factor can be forced to act as a death factor by activating an engineered receptor.

## REFERENCES

1. Quinn TP, Soifer SJ, Ramer K, Williams LT and Nakamura MC. A Receptor for Vascular Endothelial Growth Factor that Stimulates Endothelial Apoptosis. *Cancer Research*, 61:8629-8637, 2001.

## APPENDICES

None